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DNA Adduct Formation and Mutation Induction by Nitropyrenes in Salmonella and Chinese Hamster Ovary Cells: Relationships with Nitroreduction and Acetylation

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Nitrated pyrenes are environmental pollutants and potent mutagens in the Salmonella reversion assay. In this study reversion induction by 1-nitropyrene and 1,8-dinitropyrene in *Salmonella typhimurium* TA1538 and mutation induction by 1-nitropyrene in Chinese hamster ovary (CHO) cells were related to the extent of metabolism and DNA adduct formation. In suspension cultures of *Salmonella typhimurium* TA1538, 1,8-dinitropyrene was up to 40-fold more mutagenic than 1-nitropyrene, although both compounds were metabolized at similar rates with nitroreduction being the major pathway. The major metabolite formed from 1-nitropyrene after 2 hr of incubation was 1-nitrosopyrene, while 1-amino-8-nitropyrene was the major metabolite formed from 1,8-dinitropyrene. 1-Nitrosopyrene and 1-nitro-8-nitrosopyrene elicited mutation values consistent with their being intermediates in the activation pathways. However, subsequent to nitroreduction, 1,8-dinitropyrene appeared to be further activated by acetylation, while 1-nitropyrene was not. Each nitrated pyrene produced a major DNA adduct substituted at the C8-position of deoxyguanosine. Although 1,8-dinitropyrene was more mutagenic than 1-nitropyrene, both compounds induced a similar number of revertants per adduct. Incubation of 1-nitrosopyrene with CHO cells produced a rapid concentration- and time-dependent induction of mutations and the conversion of 1-nitrosopyrene to 1-aminopyrene. In contrast, 1-nitropyrene did not induce mutations and was not converted to 1-aminopyrene. Both compounds produced the same major adduct, but adduct formation by 1-nitropyrene was much lower than by 1-nitrosopyrene. These data indicate that nitroreduction and C8 deoxyguanosine adduct formation are strongly associated with mutation induction by 1-nitropyrene in Salmonella and CHO cells. With 1,8-dinitropyrene, however, mutation induction in Salmonella is dependent upon both nitroreduction and esterification

Introduction

The nitrated pyrenes comprise a group of environmental contaminants which are potent mutagens in the Salmonella reversion assay without exogenous metabolic activation. 1-Nitropyrene, which may account for up to 25% of the mutagenic activity in diesel particulate (*1–3*), produces about 500 revertants/nmole using *Salmonella typhimurium* strain TA98 (*4–7*), while the dinitropyrenes, which account for 30% or more of the mutagenic activity of diesel particulate (*8*), produce between 29,000 and 275,000 revertants/nmole in the same strain (*4–7*).

Reversion induction by 1-nitropyrene in Salmonella appears to be dependent upon bacterial nitroreduction, because the mutagenicity of this compound is greatly reduced in strains of Salmonella deficient in nitroreductase activity (*5,6*). Also, incubation of Salmonella with 1-nitropyrene results in the production of the reduced derivatives 1-aminopyrene and 1-acetylaminopyrene (*9,10*). The major DNA adduct formed from 1-nitropyrene and its intermediate reduction product 1-nitrosopyrene is *N*-(deoxyguanosin-8-yl)-1-aminopyrene (*11,12*). The structure of this adduct suggests that *N*-hydroxy-1-aminopyrene may be the reactive inter-

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mediate because DNA adducts substituted through the C8 position of guanine are formed in Salmonella by a number of *N*-hydroxy arylamines (*13–15*).

The mechanisms responsible for reversion induction in Salmonella by the dinitropyrenes are less well understood. The mutagenicity of 1,8-dinitropyrene, the most mutagenic of the dinitropyrenes, is largely independent of the bacterial nitroreductase which is involved in the activation of 1-nitropyrene (*5,6*). Incubation of 1,8-dinitropyrene with Salmonella does produce reduced metabolites and DNA binding, but the identity of some of the major metabolites and the structures of the DNA adducts formed by this compound have not been reported (*10,16,17*).

In contrast to its potent mutagenicity in Salmonella, 1-nitropyrene is nonmutagenic or questionably mutagenic in a variety of mammalian cell assay systems (*18–22*). However, 1-nitropyrene does induce sister-chromatid exchanges in CHO cells (*23*), transforms cultured mammalian cells (*24,25*), and is carcinogenic in rats (*26,27*). It is not clear whether 1-nitropyrene is a poor mutagen in mammalian cells because it produces no mutagenic DNA damage or because the genetic loci assayed are insensitive to the damage it produces.

In this study, we have attempted to answer several questions regarding the mutagenicity of nitrated pyrenes. First, in order to determine the mechanism of activation of 1,8-dinitropyrene to a reactive derivative, the identities of the major metabolites and DNA adducts formed by incubating this compound with Salmonella were determined. Second, several experiments were performed to determine why 1,8-dinitropyrene is more mutagenic than 1-nitropyrene in Salmonella: the relative mutagenicities of these two compounds were compared with the rates of formation of the individual metabolites produced from them; the number of reversions induced per adduct by 1,8-dinitropyrene was compared with the number of reversions per adduct previously determined for 1-nitropyrene (*11*), and the mutagenicities of the nitroso derivatives of 1-nitropyrene and 1,8-dinitropyrene were compared. Finally, the mutagenicity of 1-nitropyrene in mammalian cells was studied by comparing the metabolism of 1-nitropyrene and 1-nitrosopyrene by CHO cells with the mutations produced by these compounds. The DNA adducts formed by both compounds in CHO cells were also identified.

Materials and Methods

Instrumentation

High-pressure liquid chromatography (HPLC) was conducted with a Waters Associates system consisting of two 6000A pumps, a U6K injector, a 440 or a Hewlett Packard 1040A absorbance detector, an automated gradient controller and a 0.39 × 30 cm C_{18}-μBondapak 10 μm reverse-phase column. Electron impact and chemical ionization mass spectra were obtained with a Finnigan 4023 MS/DS. Radioactivity was measured with a Searle Mark III liquid scintillation counter.

Chemicals, Enzymes, Cell Cultures

1-Aminopyrene was purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified as previously described (*27*). [4,5,9,10-^{3}H]1-Nitropyrene (1055 mCi/mmole), [4,5,9,10-^{3}H]1,8-dinitropyrene (1100 mCi/mmole), [4,5,9,10-^{3}H]1-nitrosopyrene (217 mCi/mmole), 1-nitropyrene, and 1,8-dinitropyrene were obtained from the Midwest Research Institute (Kansas City, MO). 1-Acetylaminopyrene (*28*), 1-nitrosopyrene (*28*), 1-nitro-8-nitrosopyrene (*29*), 1-amino-8-nitropyrene (*29*), and 1-acetylamino-8-nitropyrene (*29*) were synthesized by the methods given in the literature.

1,8-Diaminopyrene was prepared by hydrazine reduction of 1,8-dinitropyrene. 1,8-Dinitropyrene (5 mg) was dissolved in 10 mL of chloroform and 10 mL of ethanol by gentle refluxing. Hydrazine hydrate (100 μL of 85% solution; Fisher Chemical Co., St. Louis, MO) and 10% palladium on carbon (1 mg; Alfa Products, Danvers, MA) were then added, and the mixture was refluxed for 1.5 hr. Thin-layer chromatography on silica (ethyl acetate:benzene, 2:8) indicated that the starting material was consumed and a single derivative was produced. The reaction mixture was filtered through a Celite (Fisher) bed and the filtrate was concentrated *in vacuo* to yield 1,8-diaminopyrene [*m/z* (chemical ionization): 233 $(m + 1)^+$].

N,N'-Diacetyl-1,8-diaminopyrene was synthesized by treating 3 mg of 1,8-diaminopyrene with 2 mL of acetic anhydride and 2 mL of glacial acetic acid which caused a precipitate to form almost immediately. The suspension was stirred overnight and then concentrated *in vacuo* to yield a single product as determined by HPLC [*m/z* (electron impact): 316 $(m)^+$, 274 $(m\text{-}CH_2CO)^+$, 204, 176].

N-(Deoxyguanosin-8-yl)-1-amino-8-nitropyrene was prepared by reacting 1-nitro-8-nitrosopyrene with calf thymus DNA (type I, Sigma Chemical Co., St. Louis, MO) in the presence of ascorbic acid at pH 5 in a manner similar to that used for the preparation of *N*-(deoxyguanosin-8-yl)-1-aminopyrene (*12*). Analysis of the *n*-butanol extract of the DNA hydrolyzate by HPLC indicated the presence of one major adduct. This product has been characterized by nuclear magnetic resonance and mass spectroscopy as *N*-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene. Details of this characterization will be provided in a separate communication.

Salmonella typhimurium strains TA1538 and TA98 were provided by B. N. Ames (Berkeley, CA), while strains of TA98NR and TA98/1,8-DNP_6 were obtained from H. S. Rosenkranz (Cleveland, OH). The CHO cells were CHO-K_1-BH_4 and were provided by A. W. Hsie (Oak Ridge, TN).

Salmonella Suspension Culture Incubations

Oxoid nutrient broth #2 (KC Biologicals, Lenexa, KS) was inoculated with *Salmonella typhimurium* strain TA1538, and the culture was incubated at 37°C for 12

hr with shaking. The bacteria were isolated by centrifugation (10,000*g* for 10 min) and then resuspended at a 5-fold concentration (approximately 1×10^{10} bacteria/mL) in Earle's balanced salt solution (EBSS) (GIBCO, Grand Island, NY), supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES), pH 7.3. The resuspension was accomplished by repeatedly pipetting the suspension using a 10-mL pippette, followed by vortex mixing for 3 min and then twice passing the suspension through a 1.5-in., 19-gauge needle. Aliquots of the bacterial suspension were transferred to glass Erlenmeyer flasks and treated with 3 μM [4,5,9,10-^{3}H]1-nitropyrene or 3 μM [4,5,9,10-^{3}H]1,8-dinitropyrene, both added as solutions in dimethyl sulfoxide (DMSO). The final concentration of the DMSO was <0.4%. Immediately after addition of the mutagens and after 0.5, 1.0, and 2.0 hr of incubation at 37°C, 3-mL aliquots of each of the treated and control cultures were withdrawn and diluted with 8 mL of a 50:50 mixture of EBSS and fetal bovine serum (FBS) and mixed for 30 sec. The bacteria were then isolated by centrifugation, resuspended in 8 mL of EBSS and FBS (50:50), and again isolated by centrifugation. These cell pellets were resuspended in 3 mL of EBSS and then diluted 1:10 with EBSS. To determine reversion induction, 0.1 mL aliquots of this cell suspension were mixed with 2.5 mL of molten top agar R (50 μM biotin, 50 μM L-histidine, 0.6% NaCl, 0.6% agar) and poured into plates containing 20 mL of Vogel's minimal salts agar with glucose. To determine the concentration of viable bacteria, the 1:10 dilution was further diluted $1:10^5$ with nutrient broth and 0.1-mL aliquots and were mixed with molten top agar S (100 μM biotin, 100 μM L-histidine, 0.6% NaCl, 0.6% agar) and poured into plates containing 20 mL of Vogel's agar. Assays were performed in triplicate and plates were incubated 48 hr at 37°C before the bacterial colonies were counted manually. Reversion frequencies were calculated as previously described (*12*).

Immediately after addition of the mutagens and after 0.5, 1.0, and 2.0 hr of exposure, 1-mL aliquots were withdrawn and stored at −20°C for metabolite analysis. After 0.5, 1.0, and 2.0 hr. of incubation, 20-mL aliquots were withdrawn from the 1,8-dinitropyrene incubations and the bacteria were isolated by centrifugation for use in DNA adduct analysis.

Salmonella Plate Incorporation Reversion Assays

A 0.1-mL aliquot of a 12-hr broth culture of tester strain TA98, TA98NR or TA98/1,8-DNP$_6$ was combined with 0.1 mL of DMSO or 0.1 mL of a solution of a mutagen dissolved in DMSO and 2.5 mL of top agar R. This mixture was poured into plates containing Vogel's agar, incubated and the bacterial colonies counted, as described above. The number of revertants per microgram of mutagen induced in each strain was calculated from the increasing portion of dose-response curves.

Incubations and Mutation Assays Using CHO Cells

The procedures used for exposing CHO cells to mutagens and assaying for mutations at the hypoxanthine–guanine phosphoribosyl transferase (HGPRT) locus were adapted from previously published methods (*30*). Cells were isolated from suspension culture by centrifugation at 700*g* for 7 min and resuspended at a concentration of 1×10^7 cells/mL in nutrient mixture F-12 (Irvine Scientific, Santa Ana, CA) containing 25 mM HEPES, pH 7.3. The resuspended cells were then purged with argon for 5 min. Aliquots of this cell suspension were exposed to DMSO or solutions of 1-nitropyrene or 1-nitrosopyrene in DMSO. The final concentration of DMSO was 1.0%. Aliquots from these cultures were withdrawn immediately after addition of the mutagen and after 15, 30, 60, 105, and 150 min of incubation at 37°C. The cells in these aliquots were isolated by centrifugation and resuspended in F-12. Cultures were then initiated from these cells to determine both cell survival by relative cloning ability and mutations at the HGPRT locus.

For metabolism studies, CHO cells were resuspended in F-12, treated with 20 μM [4,5,9,10-^{3}H]1-nitropyrene or [4,5,9,10-^{3}H]1-nitrosopyrene and then incubated in suspension culture at 37°C. Immediately after addition of the chemicals and after 1.0 or 2.5 hr of incubation, 0.5-mL aliquots of the incubation mixture were withdrawn and subjected to a 1-min centrifugation in a Beckman microfuge. The supernatant fluids were retained at −20°C for metabolite analysis.

For DNA adduct analysis, 2×10^8 CHO cells in 20 mL of F-12 were exposed to 13 μM [4,5,9,10-^{3}H]1-nitrosopyrene for 1 hr or 60 μM [4,5,9,10-^{3}H]1-nitropyrene for 2.5 hr. The cells were then isolated by centrifugation.

DNA Adduct Analysis

DNA was isolated from Salmonella and CHO cells as previously described (*12,31*). The DNA was enzymatically digested to nucleosides, the DNA adducts were partitioned into *n*-butanol, separated by HPLC and then quantified by scintillation counting as described elsewhere (*11*).

Metabolite Analysis

Samples of the Salmonella suspension (100 μL) were vigorously extracted with a mixture of 50 μL of chloroform and 50 μL of methanol. Following centrifugation in a Beckman microfuge, the organic phases, which contained >97% of the radioactivity, were analyzed directly by HPLC following the addition of nonradioactive UV markers. Fractions (30 sec) were collected and analyzed for radioactivity using Scintisol (Isolabs, Akron, OH). 1-Nitropyrene metabolites were separated by using a nonlinear gradient (Waters #2) of 45–75% methanol over 20 min at a flow rate of 2 mL/min. 1,8-Dinitropyrene metabolites were analyzed by using a 25-min linear gradient of from 75% A in B to 20% A in B, where A was

10 mM ammonium acetate, pH 7.1, and B was acetonitrile. The flow rate was 2 mL/min. The aqueous phase from each extraction was also analyzed by HPLC. Metabolites were identified by co-elution with the synthetic standards. The identities of the major metabolites were confirmed through comparison of their ultraviolet and visible spectra with those of the reference standards.

Metabolite analysis from CHO cell incubations were performed by injecting the supernatant fluids onto the HPLC. The HPLC analysis was identical to that used to characterize the metabolites produced during the Salmonella incubations.

Results

Incubation of *Salmonella typhimurium* TA1538 with 1-Nitropyrene or 1,8-Dinitropyrene

The toxicity and reversions resulting from incubating suspension cultures of TA1538 with 3 μM radiolabeled 1-nitropyrene or 1,8-dinitropyrene are shown in Figure 1. Both compounds produced approximately equal levels of toxicity, reducing the survival of the bacteria about 20 to 25% (Fig. 1*A*). Treatment of TA1538 with 1,8-dinitropyrene resulted in severalfold more revertants/plate (Fig. 1*B*) and revertants/10^5 viable bacteria (Fig. 1*C*) than exposure to 1-nitropyrene. After 0.5 hr of incubation, 1,8-dinitropyrene produced a 40-fold higher reversion frequency than 1-nitropyrene, while after 2 hr of incubation,this difference was reduced to 5-fold.

The relative distributions of the metabolites produced from 1-nitropyrene and 1,8-dinitropyrene during these incubations are shown in Tables 1 and 2. 1-Nitropyrene was metabolized more slowly than 1,8-dinitropyrene with 38% of the 1-nitropyrene and 28% of the 1,8-dinitropyrene remaining after 2 hr of incubation. All of the identified metabolites were produced either by nitroreduction or by nitroreduction and *N*-acetylation. Except for 1-nitrosopyrene and *N,N'*-diacetyl-1,8-diaminopyrene, all the metabolites were produced in a time-dependent manner. The major metabolite produced from 1-nitropyrene was 1-nitrosopyrene while the predominant metabolite produced from 1,8-dinitropyrene was 1-amino-8-nitropyrene. The percentage of amino-substituted derivatives produced from 1-nitropyrene at 0.5, 1.0, and 2.0 hr increased from 5 to 10,

Table 1. Metabolism of [4,5,9,10-^{3}H]1-nitropyrene by *Salmonella typhimurium TA1538*.

Retention time, min	Marker	% of total radioactivity co-eluting with marker			
		0 min[a]	30 min[a]	60 min[a]	120 min[a]
1–3[b]	—	0.8	1.0	1.7	3
9.0	1-Acetylamino-pyrene	1	2	5	5
10.0	1-Aminopyrene	1	3	5	8
11.0	—	1	2	3	5
18.0	1-Nitropyrene	86	65	49	38
19.5	1-Nitrosopyrene	0[c]	0[c]	15	14

[a] Incubation time. The "0 min" sample was taken immediately after addition of [4,5,9,-10^{3}H]1-nitropyrene.
[b] Void volume.
[c] Could not be resolved from 1-nitropyrene.

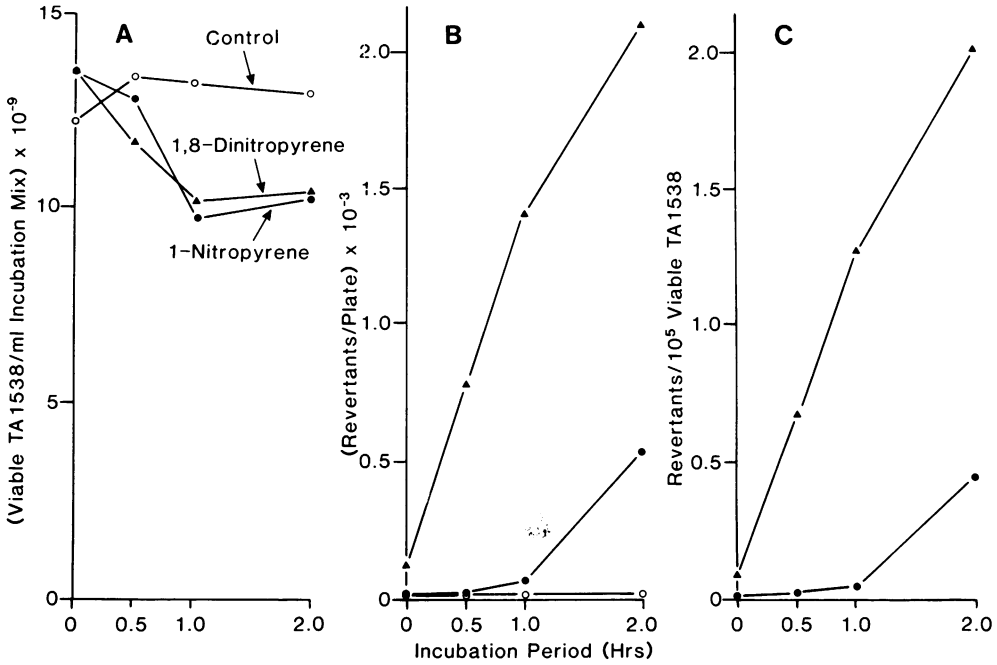

FIGURE 1. Plots of (*A*) bacterial survival, (*B*) revertants/plate, and (*C*) revertants/10^5 viable bacteria produced in suspension cultures of *Salmonella typhimurium* TA1538 exposed for up to 2 hr to DMSO (control) or 3 μM [4,5,9,10-^{3}H]1,8-dinitropyrene or 3 μM [4,5,9,10-^{3}H]1-nitropyrene.

Table 2. Metabolism of [4,5,9,10-^{3}H]1,8-dinitropyrene by *Salmonella typhimurium* TA1538.

Retention time min	Marker	Total radioactivity co-eluting with marker, % 0 min[a]	30 min[a]	60 min[a]	120 min[a]
1–3[b]	—	0.2	1.1	1.1	1.9
5.0	1-Acetylamino-8-aminopyrene	<0.1[c]	<0.1	<0.1	<0.1
7.0	1,8-Diaminopyrene	<0.1	0.2	0.4	0.6
8.0	*N,N'*-Diacetyl-1,8-diaminopyrene	<0.1	0.1	0.2	0.1
9.5	1-Acetylamino-8-nitropyrene	0.1	0.3	0.6	1.0
14.0	1-Amino-8-nitropyrene	9.4	41.0	56.2	66.0
17.0	1,8-Dinitropyrene	88.0	55.0	38.5	27.6
19.0	1-Nitro-8-nitrosopyrene	<0.1	<0.1	<0.1	<0.1

[a] Incubation time. The "0 min" sample was taken immediately after addition of [4,5,9,10-^{3}H]1,8-dinitropyrene.
[b] Void volume.
[c] No significant radioactivity.

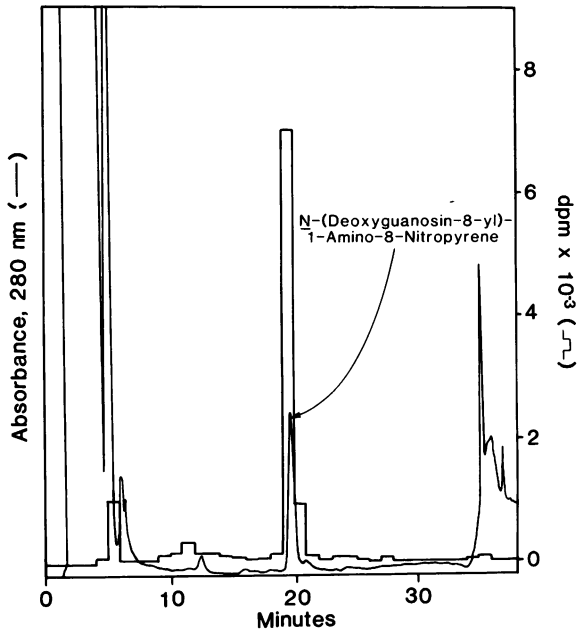

FIGURE 2. Reverse-phase high-pressure liquid chromatographic profile of the nucleoside DNA adducts isolated from *Salmonella typhimurium* TA1538 exposed to [4,5,9,10-^{3}H]1,8-dinitropyrene. Nonradioactive *N*-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene was included as a UV-absorbing marker.

to 13%, respectively, while the amino-substituted derivatives of 1,8-dinitropyrene accounted for 42, 57, and 68% of the total radioactivity at these time points. Both compounds were also metabolized to unknown products which migrated with the void volume. In addition, 1-nitropyrene was converted to an unknown derivative (R_t = 11.0 min) which accounted for 5% of the total radioactivity after 2.0 hr.

Chromatographic analysis of the mutagen-deoxyribonucleoside adducts obtained from Salmonella strain TA1538 exposed to [4,5,9,10-^{3}H]1,8-dinitropyrene indicated that most of the radioactivity migrated as a single peak (Fig. 2). This major DNA adduct co-eluted with an *N*-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene marker at 20 min. Minor radioactive peaks migrated at 5 min and 9 min. The number of DNA adducts produced by 1,8-dinitropyrene in TA1538 was compared with the number of reversions induced by these treatments. Based on three determinations, 1 DNA residue/10^5 nucleotides resulted in approximately 23 revertants/10^6 viable bacteria.

Reversions Induced by 1-Nitrosopyrene and 1-Nitro-8-nitrosopyrene

The reversions induced by 1-nitropyrene and 1,8-dinitropyrene and their respective nitroso derivatives were compared in the plate incorporation assay using Salmonella strains TA98, TA98NR and TA98/1,8-DNP$_6$ (Table 3). 1-Nitropyrene was approximately 10-fold less mutagenic in TA98NR than in either TA98/1,8-DNP$_6$ or TA98. 1-Nitrosopyrene was equally mutagenic in all three strains but approximately 20-fold more mutagenic than was 1-nitropyrene in either TA98 or TA98/1,8-

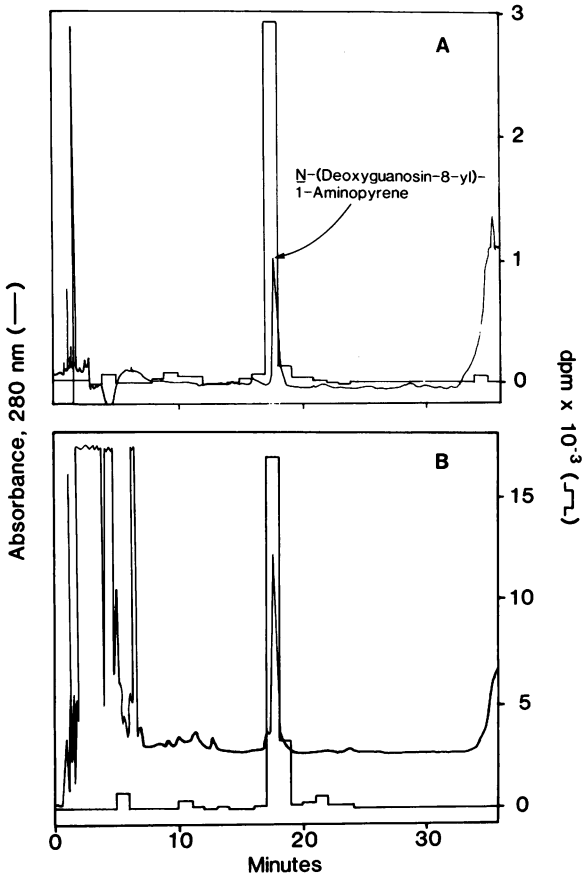

FIGURE 3. Reverse-phase high-pressure liquid chromatographic profiles of the nucleoside DNA adducts isolated from CHO cells exposed to (*A*) [4,5,9,10-^{3}H]1-nitropyrene or (*B*) [4,5,9,10-^{3}H]1-nitrosopyrene. Nonradioactive *N*-(deoxyguanosin-8-yl)-1-aminopyrene was included in both separations as a UV-absorbing marker.

Table 3. Reversions produced by 1-nitropyrene, 1,8-dinitropyrene, and their nitroso derivatives in *Salmonella typhimurium* TA98, TA98NR, and TA98/1,8-DNP_6.

Compound	Revertants/μg/plate		
	TA98	TA98NR	TA98/1,8-DNP_6
1-Nitropyrene	2107	208	1907
1-Nitrosopyrene	41960	38120	42680
1,8-Dinitropyrene	1411200	1192800	22080
1-Nitro-8-nitrosopyrene	264740	274260	5320

DNP_6. Compared with its mutagenicity in TA98, the mutagenicity of 1,8-dinitropyrene was slightly reduced in TA98NR and greatly reduced in TA98/1,8-DNP_6. 1-Nitro-8-nitrosopyrene was about equally mutagenic in TA98 and TA98NR and was much less mutagenic in TA98/1,8-DNP_6. In TA98, 1-nitro-8-nitrosopyrene was approximately 5-fold less mutagenic than 1,8-dinitropyrene.

Incubation of CHO Cells with 1-Nitropyrene or 1-Nitrosopyrene

Table 4 shows the mutations and toxicity resulting from incubating 1-nitropyrene or 1-nitrosopyrene with suspension cultures of CHO cells for up to 150 min. Neither 20 μM nor 60 μM 1-nitropyrene produced any significant reduction in cellular cloning ability or induction of mutations at the HGPRT locus. Incubations conducted with 1-nitrosopyrene, however produced time- and concentration-dependent reductions in cellular survival and increases in mutation frequency. Although the maximum responses to 1-nitrosopyrene occurred after 60-min (20 μM exposure) and 105-min (60 μM exposure) incubations, substantial levels of toxicity and mutation induction were found in cells assayed immediately after addition of the compound. In cells assayed after the longest incubation periods, the toxicities and mutation frequencies were less than the maximum values.

The metabolites produced by incubating [4,5,9,10-^{3}H]1-nitropyrene or [4,5,9,10-^{3}H]1-nitrosopyrene were determined by analyzing the media by HPLC. Incubation of 1-nitropyrene with CHO cells for 2.5 hr resulted in no detectable metabolism. However, 1-nitrosopyrene was rapidly converted to 1-aminopyrene in the presence of CHO cells. Immediately after addition of the compound, approximately 32% of the radioactivity added to the cultures migrated with the 1-aminopyrene marker and essentially no 1-nitrosopyrene was detected after 1 hr of incubation.

DNA adduct analysis was performed on cultures of CHO cells exposed to 60 μM [4,5,9,10-^{3}H]1-nitropyrene for 2.5 hr or 13 μM [4,5,9,10-^{3}H]1-nitrosopyrene for 1 hr. The chromatographic profiles of the DNA adducts isolated from these cultures are shown in Figure 3. Exposure to either compound resulted in the production of a single major DNA adduct which migrated with an *N*-(deoxyguanosin-8-yl)-1-aminopyrene marker. Exposure to 1-nitropyrene produced 1.9 adducts/10^6 nucleotides, 13 mutations/10^6 viable cells (vs. a solvent control value of 16 mutations/10^6 viable cells) and a relative cloning efficiency of 99.3%, while the 1-nitrosopyrene produced 88 adducts/10^6 nucleotides, 268 mutations/10^6 viable cells (vs. a solvent control value of 18 mutations/10^6 viable cells) and a relative cloning efficiency of 66.5%.

Discussion

Previous studies have indicated that the mutations induced by 1-nitropyrene in the *Salmonella typhimurium* reversion assay were produced as a consequence of bacterial nitroreduction. The results of this study, including the identities of the metabolites formed from 1,8-dinitropyrene, the mutagenicity of 1-nitro-8-nitro-

Table 4. Toxicity and mutations at the HGPRT locus produced by 1-nitropyrene and 1-nitrosopyrene in CHO cells.

Compound	Concentration, μM	Incubation time, min	Relative cloning ability, %	Mutations/10^6 cells
Experiment 1				
None (DMSO control)	0	60	100	25
		150	100	26
1-Nitropyrene	20	60	90.2	21
		150	103	9
1-Nitrosopyrene	20	0[a]	86.0	181
		30	75.4	245
		60	73.3	253
		105	74.1	214
		150	88.2	190
Experiment 2				
None (DMSO control)	0	60	100	17
		150	100	15
1-Nitropyrene	60	60	101	12
		150	123	17
1-Nitrosopyrene	60	0	51.5	308
		15	10.3	438
		30	6.9	474
		60	4.5	508
		105	3.5	663
		150	4.0	477

[a] Sample taken immediately after addition of compound.

sopyrene and the structure of the major DNA adduct formed by 1,8-dinitropyrene, indicate that nitroreduction also plays a major role in the mutagenic response of 1,8-dinitropyrene in Salmonella.

All the identified metabolites formed from 1,8-dinitropyrene in incubations with Salmonella TA1538 (Table 2) were the products of nitroreduction: either amino or *N*-acetylamino derivatives. In fact, every possible aminonitro, diamino, and *N*-acetylated amino derivative of 1,8-dinitropyrene was isolated in this study except for 1-acetylamino-8-aminopyrene. This derivative, however, has been found in other similar incubations with Salmonella (data not presented). The acetylamino derivatives isolated in this study may correspond to the unknown metabolites found by Bryant et al. (*17*) in incubations of TA98 with 1,8-dinitropyrene. The production of amino derivatives roughly paralleled the induction of reversions produced as a consequence of these incubations.

Nitroso or *N*-hydroxyamino derivatives were not detected as metabolites of 1,8-dinitropyrene, but the production of amino derivatives implies the transient formation of these species. The mutagenicity of 1-nitro-8-nitrosopyrene in Salmonella strains TA98, TA98NR, and TA98/1,8-DNP_6 (Table 3) was consistent with this compound being an intermediate in the activation of 1,8-dinitropyrene to a reactive derivative. Relative to its mutagenicity in TA98, the mutagenicity of 1,8-dinitropyrene was slightly reduced in TA98NR and greatly reduced in TA98/1,8-DNP_6. TA98NR is deficient in a nitroreductase activity (*32–33*), while TA98/1,8-DNP_6 is deficient in an esterificase which apparently *O*-acetylates certain aryl hydroxylamines (*34–36*). 1-Nitro-8-nitrosopyrene, which is a presumed product of nitroreduction, was equally mutagenic in TA98 and TA98NR, and, like its parent, it was a relatively weak mutagen in TA98/1,8-DNP_6. The low mutagenicity of 1-nitro-8-nitrosopyrene relative to 1,8-dinitropyrene in TA98 may be a function of adding an easily reduced compound exogenously to the Salmonella. The reactive intermediates produced from 1-nitro-8-nitrosopyrene may be less effective in damaging DNA under these conditions than if they were produced intracellularly from 1,8-dinitropyrene.

The major DNA adduct formed from 1,8-dinitropyrene in suspension cultures of Salmonella TA1538 was *N*-(deoxyguanosin-8-yl)-1-amino-8-nitropyrene. A similar adduct, *N*-(deoxyguanosin-8-yl)-1-aminopyrene, has been reported as the major adduct formed from incubating TA1538 with 1-nitropyrene (*11*). Adducts of this type, substituted through the C8 of guanine, are formed in Salmonella by many *N*-hydroxy arylamines (*13–15*). The structure of this adduct from 1,8-dinitropyrene also indicates that most of the DNA damage was a result of reduction of only one of the two nitro groups on the molecule. This observation is consistent with the results of Bryant et al. (*17*), which indicate that 1-amino-8-nitropyrene is a poor mutagen in Salmonella.

Although both 1-nitropyrene and 1,8-dinitropyrene are apparently metabolized to similar *N*-hydroxyamino intermediates, 1,8-dinitropyrene is considerably more mutagenic in Salmonella than is 1-nitropyrene (*4–7*). In the present study, 1,8-dinitropyrene was about 700 times more mutagenic than 1-nitropyrene in the Salmonella plate incorporation assay using TA98 (Table 3) and from 5 to 40 times more mutagenic when TA1538 were treated in suspension culture before assaying for reversions (Fig. 1). The difference in the mutagenicities of these two compounds in the suspension culture experiments was not solely due to differences in the extent of metabolism. 1,8-Dinitropyrene was metabolized at a rate that was only slightly faster than that of 1-nitropyrene. A similar observation has been reported by Bryant et al. (*17*). The difference in the mutagenicities of 1,8-dinitropyrene and 1-nitropyrene also cannot be solely accounted for by a difference in the number of reversions produced per adduct by these two compounds. Howard et al. (*11*) found that approximately 17 revertants/10^6 surviving TA1538 were induced by 1-nitropyrene at a level of 1 DNA adduct/10^5 nucleotides while in this study we have found that 1,8-dinitropyrene induced about 23 revertants/10^6 viable bacteria at the same level of DNA binding.

The results of our study do suggest two possible explanations for why 1,8-dinitropyrene is a more potent mutagen than 1-nitropyrene in Salmonella. Even though the rates of metabolism of these two compounds were similar, the nature of the metabolites formed were quite different. All the identified metabolites of 1,8-dinitropyrene were amino derivatives (Table 2), which implies that *N*-hydroxy derivatives, the derivatives most closely associated with the formation of DNA adducts, were transient intermediates. The major metabolite produced from 1-nitropyrene was 1-nitrosopyrene (Table 1), presumably a premutagenic species which must be further reduced to form an *N*-hydroxy derivative. Chemically, 1,8-dinitropyrene is more easily reduced than 1-nitropyrene (*37*), which may account for its more extensive conversion to amines. It is also possible that the reductases involved in 1,8-dinitropyrene metabolism are more efficient in reduction to amine derivatives than are the reductases responsible for the metabolism of 1-nitropyrene. Assuming amine formation is indicative of *N*-hydroxy arylamine formation, incubation for 0.5, 1.0 and 2.0 hr with Salmonella produced about 8, 6, and 5 times more *N*-hydroxyarylamine from 1,8-dinitropyrene than from 1-nitropyrene. Such differences may partially account for the 40-, 20-, and 5-fold differences in mutagenicity at these same time points.

Another difference in the metabolism of these compounds which may affect their overall mutagenicities in Salmonella is evident in the data presented in Table 3. For maximum reversion induction, 1,8-dinitropyrene required the esterificase activity which is reduced in TA98/1,8-DNP_6. The mutagenicity of 1-nitropyrene in TA98 was not dependent on this esterificase. Presumably, this esterificase *O*-acetylates the *N*-hydroxyarylamine generated from 1,8-dinitropyrene (*34–36*). It is

possible that this ester is much more capable of producing DNA damage than *N*-hydroxy-1-amino-8-nitropyrene and, perhaps, *N*-hydroxy-1-aminopyrene. In TA98/1,8-DNP$_6$, where extensive *O*-acetylation presumably does not occur, 1,8-dinitropyrene is only 10-fold more mutagenic than 1-nitropyrene.

In CHO cells, both 1-nitropyrene and 1-nitrosopyrene formed the same major DNA adduct, *N*-(deoxyguanosin-8-yl)-1-aminopyrene. This was the same major adduct formed by these compounds in Salmonella (*11,12*), and its structure suggests that its formation was dependent upon the reduction of 1-nitropyrene and 1-nitrosopyrene to *N*-hydroxy-1-aminopyrene. Mutation induction and DNA adduct formation by 1-nitrosopyrene in CHO cells were closely associated with conversion of this compound to 1-aminopyrene. Incubations conducted with 1-nitropyrene resulted in no detectable amine formation and no induction of mutations at the HGPRT locus although a low level of adducts was formed.

Conclusions

Nitroreduction of 1-nitropyrene in Salmonella and CHO cells, and nitroreduction of 1,8-dinitropyrene in Salmonella were closely associated with mutation induction by these compounds. The structures of the DNA adducts produced by 1-nitropyrene and 1,8-dinitropyrene in Salmonella suggest that *N*-hydroxyarylamine derivatives were the proximate mutagenic species. The relatively low mutagenicities of 1,8-dinitropyrene and its intermediate reduction product, 1-nitro-8-nitrosopyrene, in TA98/1,8-DNP$_6$ compared to TA98 suggest that *O*-acetylation of the *N*-hydroxy arylamine derivative was also involved in the mutagenic response of 1,8-dinitropyrene in Salmonella. This involvement of *O*-acetylation in the metabolism of 1,8-dinitropyrene, combined with the relatively rapid conversion of 1,8-dinitropyrene to an *N*-hydroxyarylamine derivative may explain why 1,8-dinitropyrene is a better mutagen than 1-nitropyrene in Salmonella.

Comparison of the number of mutations, metabolism and extent of DNA adduct formation produced by 1-nitropyrene and 1-nitrosopyrene in CHO cells suggests that these cells are largely deficient in the nitroarene reductase activity for 1-nitropyrene. Although 1-nitropyrene was nonmutagenic in CHO cells, it did produce potentially mutagenic DNA damage. This observation suggests that mutation induction at the HGPRT locus may be relatively inefficient in detecting the DNA damage produced by nitrated pyrenes or that CHO cells may be very proficient in repairing the DNA damage produced by these compounds.

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